Remarks/Arguments

Reconsideration of the above-identified application in view of the present remarks is respectfully requested. Below is a discussion of the 35 U.S.C. §112, first paragraph rejection of claims 4, 6-18, 15, 26, 27, and 28-39. No amendments have been made to the claims.

1. 35 U.S.C. §112, first paragraph rejection of claims 4, 6-8, 15, 26, 27 and 28-39

Claims 4, 6-8, 15, 26 and 27 stand rejected and claims 28-39 are newly rejected under 35 U.S.C. 112, first paragraph, "because the specification fails to provide an enablement for the full scope of the claimed invention." The Office Action maintains that Kerbel, *Cancer & Metastasis Rev*. 17:301-304, 1999 (Hereinafter, "Kerbel 1"), Vieweg *et al*., *Cancer Investig*. 13(2):193-201, 1995 (hereinafter, "Vieweg"), and Hoffman, *Invest. New Drugs* 17: 343-360, 1999 (hereinafter, "Hoffman") demonstrate that "orthotopically transplanted tumors do not necessarily recapitulate the 'encouraging' responses of their ectopically (usually subcutaneous) grown counterparts, and that the animal model exemplified in the instant specification, *i.e*., subcutaneously-growing human cancer cell lines in immunedeficient mice, do not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity."

Applicants respectfully submit that the amount of direction or guidance disclosed in the present specification is sufficient to enable the skilled artisan to make and use the method recited in claims 4, 6-8, 15, 26-27, and 28-39 using only routine experimentation.

"[T]o be enabling, the specification...must teach those skilled in the art how to make and use *the full scope of the claimed invention* without 'undue experimentation.'" *Id.* at 1561 (emphasis added), *quoted in Genentech, Inc. V. Novo Nordisk A/S*, 108 F.3d 1361, 1365 (Fed. Cir. 1997). Thus, "there must be sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the invention as broadly as it is claimed." *In re Vaeck*, 947 F.2d 488, 496 & n. 23 (Fed. Cir. 1991), *quoted in Enzo Biochem, Inc. V. Calgene, Inc.*, 188 F.3d 1362, 1374 (Fed. Cir. 1999). Some experimentation, even a considerable amount, is not "undue" if, *e.g.*, it is merely routine, or if the specification provides a reasonable amount of guidance as to the direction in which the experimentation should proceed. *See In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988).

Facts that should be considered in determining whether a specification is enabling include: (1) the quantity of experimentation necessary to practice the invention; (2) the amount of direction or guidance presented; (3) the presence or absence of working examples; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims. *In re Wands,* 858 F.2d 731, 737 (Fed. Cir. 1988).

As discussed in Applicants' previous response, the specification of the present application provides guidance and direction to the skilled artisan commensurate with the scope of the claims. The specification of the present application teaches that apoptosis can be induced in prostate cancer cells or breast

cancer cells by delivering and expressing a nucleic acid encoding human KChAP protein (in a viral vector) via intratumoral injection. The specification notes that the present method is especially useful for treating a patient with an epithelial carcinoma, such as breast cancer or prostate cancer (¶0047). For example, the specification notes that apoptosis may be induced in cancer cells, particularly prostate cancer cells, by introducing a KChAP protein in the cell (¶0070). The specification also notes that polynucleotides comprising a coding sequence for KChAP protein can include a promoter that permits expression of the protein (¶0062). Additionally, the specification notes that viral vectors may be used to deliver the KChAP polynucleotide to the cell (¶0063). Further, the specification notes that delivery of the KChAP polynucleotide may be via intratumoral injection (¶0075).

The specification of the present application also includes several working examples demonstrating that delivery and expression of a nucleotide sequence encoding human KChAP protein induces apoptosis in human prostate cancer and breast cancer cells. Example 1 of the specification demonstrates that delivery and expression of a nucleotide sequence encoding KChAP induces apoptosis in LNCaP cells, which are a prostate cancer cell line containing native p53 protein. Example 2 of the specification demonstrates that delivery and expression of a nucleotide sequence encoding KChAP induces apoptosis in Du145 cells, which are a prostate cancer cell line containing mutated p53 protein. Example 3 of the specification demonstrates that *in vivo* growth of subcutaneous implants of human prostate cancer cells is inhibited by increasing intracellular levels of KChAP. Example 4 of the specification demonstrates that delivery and expression of a nucleotide

sequence encoding human KChAP protein induces apoptosis in mammary epithelial cancer cells (*i.e.*, MCF-7 cells). Taken together, Examples 1-4 demonstrate that delivery and expression of a nucleotide sequence encoding human KChAP protein induces apoptosis in both prostate cancer cells and breast cancer cells.

Additionally, in contrast to the Office Action's assertions, the animal models used in the present application do sufficiently represent clinical cancer to enable a skilled artisan to practice the invention. The author of the Kerbel 1 article cited in the Office Action also authored a more recent article, Kerbel, Cancer Biology & Therapy, 2:4: Suppl., S134-S139, 2003 (hereinafter, "Kerbel 2"), that 1) discusses the pros and cons of different animal models of human carcinoma, and 2) specifically that the xenograft animal model used to support the claimed invention is sufficient to reflect human carcinoma, and 3) that the alternate models argued in the Office Action as a preferred models to the Applicants' xenografts model have drawbacks.

Kerbel 2 states: "Human tumor xenografts – even non metastatic ectopic/subcutaneous 'primary' tumor transplants – can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinical equivalent or 'rational' drug doses" (Kerbel 2, p.S134). Kerbel 2 further states that the skepticism levied at human tumor xenograft models based on the disparity between some results seen in mice versus result from clinical trial is not always warranted because "a crucial and fundamental difference between virtually all published experimental mouse therapy studies and corresponding clinical trials" is that "in most phase I, II and II clinical trials the patients being treated have advanced, high-volume metastatic disease whereas

most mouse studies do not test the effects of therapy on advanced metastatic disease, but rather on primary tumor transplant or spontaneously rising primary tumor, or microscopic, low-volume metastatic disease" (p. S137). In addition, Kerbel 2 states that "one of the limitations of many of the current transgenic oncomouse models" is that "they usually do not spontaneously metastasize" (p.S139). "The wisdom of the rush towards exclusive use of much more expensive transgenic oncomouse models for drug therapy testing *can be questioned*, especially when such tumors fail to express the most critical element of malignant disease – ability to metastasize, and the fact that less expensive transplantable tumor models are available which work – if used appropriately" (Kerbel 2, p139, emphasis added). Therefore, Applicants respectfully submit that, in view of Kerbel 2 and the inherent limitations present in all murine models of human carcinoma, the animal model used to support the claimed invention is sufficient to reflect human carcinoma.

The Office Action also states that, "the working examples cited by Applicants are not commensurate in scope with the claimed invention and are directed to using cell lines, that in turn fail to reflect the issues raised for normal breast and prostate tumors, comprising a mixture of normal and transformed cells." As discussed above, according to Kerbel 2, the animal model used by the Applicants are clinically relevant. In addition, the specification also teaches that KChAP induces apoptosis in both p53 wild-type and p53 mutant cells, thereby demonstrating efficacy in heterogeneous cancer cell types. Therefore, Applicants respectfully submit that the examples are sufficiently commensurate in scope with the claimed invention and,

therefore, do enable the skilled artisan to make and use the method in the claims using only routine experimentation.

The Office Action references Wang, *Eur. J. Physiol*. 448:274-286, 2004 (hereinafter, "Wang") and Shieh *et al*., Pharmacol. Rev. 52:557-93, 2000 (hereinafter, "Shieh"), to support its assertion of the unpredictability with regard to K+ channel expression. The Office Action maintains the pending claims are not enabled because of the "paradox that enhancement of K+ channel activity can facilitate not only tumor cell apoptosis but also tumor cell proliferation, especially in a tumor mass comprising a mixed cell population, bringing into question the validity of the claimed method as a therapeutic." However, Applicants respectfully submit that the Examiner has improperly broadened the claimed invention to include the expression of all K+ channels in general. The claimed invention does not encompass expression of all K+ channels in general. Rather, the claimed invention is directed towards the expression of a specific protein – KChAP. As discussed in more detail below, KChAP boosts expression of a subset of K+ channels (not all K+ channels in general), and is a pro-apoptotic regulator of prostate and breast cancer cells, not a pro-ongogenic. Thus, the Office Actions assertion of the unpredictability with regard to K+ channel expression is misplaced. The expression of KChAP is indeed predictable.

First, the Office Action cites to Wang in support of its assertion of the unpredictability of the claimed invention. "K+ channels favor tumor cell proliferation; therefore, inhibition of K+ channel function or down-regulation of K+ channel expression should inhibit tumorigenesis. On the other hand, K+ channels also

promote apoptotic cell death enhancement of K+ channel activity can facilitate not only tumor cell apoptosis but also tumor cell proliferation. This apparent paradox confounds the manipulation of K+ channel function and/or expression as an option for the treatment of cancers." (Wang, pp.281-282 bridging).

On the contrary, Wang actually distinguishes KChAP function from the above general statement with regard to K+ channel function. "KChAP boosts protein expression of a *subset* of K+ channels" (Wang, p279, column 2, emphasis added). Therefore, Wang actually teaches that the function of KChAP is *specific* to a subset of K+ channels, indicating a more *specific* and *predictable* result as opposed to a broad spectrum K+ channel activator. Furthermore, Wang also states that "[c]onsistent with its pro-apoptotic properties, KChAP prevents the growth of DU145, another prostate cancer cell line, and LNCaP tumour xenografts in nude mice, indicating that infection with Ad/KChAP might represent a novel method of cancer treatment" (p279-80). Thus, Wang only references *KChAP* as a *pro-apoptotic* regulator and makes **no** reference to KChAP being a *pro-oncogenic*. Therefore, the "paradox" presented by Wang is not present in the claimed invention because the claims specifically identify KChAP and KChAP does induce apoptosis in pancreatic and breast cancer cells as claimed.

Second, to further support its assertion of the unpredictability of the claimed invention, the Office Action cites Shieh as a review of the prior art with regard to K+ channels as potential therapeutic targets at the time of the invention. "[Shieh] describe KChAP as a chaperone protein, or auxullary (*sic*) factor, regulating the function and expression of the Kv Channels, such as Kv2.1, Kv1.3 and Kv4.3, and

state that given the diversity of K+ channel subunits, understanding the composition of channel complexes *in vivo* remains a challenge" (Office Action p. 4).

However, this statement suggests that KChAP is known to bind to more Kv proteins than Kv2.1, Kv1.3, and Kv4.3, which is simply not true. Kuryshev, *et al.*, Am J Physiol Cell Physiol, 278: C931-41, 2000 (hereinafter, "Kuryshev"), is referenced by Shieh for the above statement. Kuryshev demonstrate that, in a test for KChAP/Kv channel interactions, Kv2.1, Kv1.3, and Kv4.3 were the *only* proteins found to interact with KChAP out of 11 tested Kv channel proteins (Kuryshev, Fig. 2.). "Our results demonstrate that KChAP modulates the functional expression of *specific* Kv channels" (Kuryshev, p.C939, column 2, emphasis added). Therefore, Kuryshev indicates that KChAP interacts *specifically* and *predictably* with three Kv channel proteins.

Furthermore, the statement used in the Office Action with reference to Shieh "understanding the composition of channel complexes in vivo remains a challenge" (Shieh, p566, column 1), is used out of context and does not actually support the Office Action's assertion of the unpredictability of the claimed invention. Shieh makes this statement in a larger discussion regarding combinations of all K+ channels in all tissue types (*Id.)* stating, "[g]iven the diversity of K+ channel subunits and the potential to vary the constituents to form diverse α- α or α-β heteromeric channel complexes to alter expression, cellular targeting, and biophysical and pharmacological properties in native cell types, understanding the precise composition of channel complexes in vivo remains a challenge". Applicants, however, claim expressing KChAP to induce apoptosis in two subsets of cancer,

breast and prostate, not the activation of all K+ channels found on all cells. Therefore, understanding the composition of KChAP channel complexes is not a challenge given their specificity and predictability as discussed above by Kuryshev Fig.2.

Next, the Office Action continues to quote Shieh in support of its rejection of the claims: "K+ channel activities play important roles in signal transduction pathways leading to proliferation, differentiation and cell fusion, that enhancement of current is directly involved in apoptosis and oncogenesis, and that overexpression of rEAG K+ channels in Chinese hamster ovary or NIH 3T3 cells induces significant features characteristic of malignant transformation." However, Applicants fail to see the relevance of these statements because, in discussing K+ induced proliferation, Shieh does not make any specific references to KChAP or the three specific K+ channel proteins with which KChAP was known to interact (with reference to Kuryshev, Fig 2). In addition, the Office Action does not cite a reference to suggest that rEAG interacts with KChAP. Furthermore, Chinese hamster ovary cells and NIH 3T3 cells are note breast cancer cells or prostate cancer cells.

The Office Action concludes that "the prior art of Shieh *et al.* highlights the unpredictability with regard to targeting K+ channel expression, and together with the post-filing art of Wang *et al.* argue against the overexpression of K+ channels, as such is known to promote malignancy." As discussed before, Applicants suggest that the Office Action has improperly broadened the claimed invention to include the overexpression or activation of all K+ channels. Applicants respectfully submit that the claimed invention does not claim the overexpression of K+ channels generally,

but instead claims the expression of KChAP (Claims 4 and 34, "…delivering to and expressing in said cells a nucleic acid comprising: i) a nucleotide sequence encoding human KChAP protein…"). In addition, as discussed above, because Kureyshev teaches that KChAP specifically interacts with Kv2.1, Kv1.3, and Kv4.3, the activity of KChAP is *predictable*. Therefore, the expression of KChAP in prostate and breast cancer cells do yield predictable results.

Moreover, as discussed in Applicants previous response, the Office Action's apparent position that the specification cannot teach how to use the claimed method unless it teaches solutions to all the problems in the field of cancer therapy is contrary to controlling case law. *See, e.g., In re Brana,* 51 F.3d 1560, 1568 (Fed. Cir. 1995).

In *Brana,* the claims were directed to compounds disclosed as anticancer agents. *Id.* at 1562. The USPTO rejected the claims as non-enabled, *id.* at 1563-64, despite working examples in Brana's specification showing treatment of cancer in a mouse model. *Id.* at 1562-63. The USPTO argued that the results of the mouse testing "are not reasonably predictive of the success of the claimed compounds for treating cancer in humans." *Id.* at 1567. The court concluded that this position "confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption." *Id.* The *Brana* court held that "[u]sefulness in patent law, and in particular in the context of pharmaceutical inventions, necessarily includes the expectation of further research and development. The stage at which an invention in

this field becomes useful is well before it is ready to be administered to humans." *Id* at 1568.

Here, the claims are simply directed to a method of inducing apoptosis in human prostate cancer or breast cancer cells, and Applicants' specification provides several working examples demonstrating just that both *in vivo* and *in vitro*. The Examiner has discounted the specification's working examples because "the animal model exemplified in the instant specification, i.e. subcutaneously-growing human cell lines in immunodeficient mice, do[es] not sufficiently represent clinical cancer, especially with regard to metastasis and drug sensitivity". However, Applicants have discussed that Kerbel 2 teaches that xenografts mouse models are acceptable clinical cancer treatment models that yield results that can be indicative of clinical efficacy. Furthermore, enablement includes an expectation of further research and development. Thus, enablement is not precluded even if the claims encompass methods, such as prostate and breast cancer therapy that have not yet overcome all the obstacles to their clinical use.

The Office Action has not established that undue experimentation would have been required to practice the *claimed* method; specifically, a method of inducing apoptosis in human prostate cancer or breast cancer cells. Therefore, the amount of direction or guidance disclosed in the specification is sufficient to enable the skilled artisan to make and use the methods of claims 4, 6-8, 15, 26-27, and 28-39 using only routine experimentation.

Accordingly, Applicants respectively submit that claims 4, 6-8, 15, 26-27, and 28-39 are enabled by the present application, and request that the 35 U.S.C. §112, first paragraph, rejection of these claims be withdrawn.

In view of the foregoing, it is respectfully submitted that the present application is in a condition of allowance and allowance of the present application is respectfully requested.

Please charge any deficiency or credit any overpayment in the fees for this matter to our Deposit Account No. 20-0090.

Respectfully submitted,

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Models of Anti-Cancer Therapy

Human Tumor Xenografts as Predictive Preclinical Models for Anticancer Drug Activity in Humans

Better Than Commonly Perceived—But They Can Be Improved

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ABSTRACT

It is not uncommon for new anti-cancer drugs or therapies to show highly effective, and sometimes even spectacular anti-cancer treatment results using transplantable tumors in mice. These models frequently involve human tumor xenografts grown subcutaneously in immune deficient hosts such as athymic (nude) or severe combined immune deficient (SCID) mice. Unfortunately, such preclinical results are often followed by failure of the drug/therapy in clinical trials, or, if the drug is successful, it usually has only modest efficacy results, by comparison. Not surprisingly, this has provoked considerable skepticism about the value of using such preclinical models for early stage in vivo preclinical drug testing. As a result, a shift has occurred towards developing and using spontaneous mouse tumors arising in transgenic and/or knockout mice engineered to recapitulate various genetic alterations thought to be causative of specific types of respective human cancers. Alternatively, the opinion has been expressed of the need to refine and improve the human tumor xenograft models, e.g., by use of orthotopic transplantation and therefore promotion of metastatic spread of the resultant 'primary' tumors.

Close inspection of retrospective and prospective studies in the literature, however, reveals that human tumor xenografts—even non metastatic ectopic/subcutaneous 'primary' tumor transplants—can be remarkably predictive of cytotoxic chemotherapeutic drugs that have activity in humans, when the drugs are tested in mice using pharmacokinetically clinically equivalent or 'rational' drug doses. What may be at variance with clinical activity, however, is the magnitude of the benefit observed in mice, both in terms of the degree of tumor responses and overall survival. It is argued that this disparity can be significantly minimized by use of orthotopic transplant/metastatic tumor models in which treatment is initiated after the primary tumor has been removed and the distant metastases are well established and macroscopic—i.e., the bar is raised and treatment is undertaken on advanced, high volume, metastatic disease. In such circumstances, survival should be used as an endpoint; changes in tumor burden using surrogate markers or micro-imaging techniques can be used as well to monitor effects of therapies on tumor response. Adoption of such procedures would more accurately recapitulate the phase I/II/III clinical trial situation in which treatment is initiated on patients with advanced, high-volume metastatic disease.

INTRODUCTION

One of the greatest challenges faced by developers of new drugs and treatment strategies for cancer is the obvious need to test them in preclinical in vivo models that have a good probability of being predictive of similar activity in humans. For more than half a century the laboratory mouse has been the primary species in which experimental cancer treatments have been tested. Until about 25 years ago syngeneic transplantable mouse tumors were used most commonly for such preclinical therapy studies, and still are, especially for immunotherapy experiments in which an intact immune system is required. The discovery that human tumor cell lines, and sometimes even primary biopsy human tumor specimens, can give rise to progressively growing, and potentially lethal cancers in immune deficient mice gradually resulted in a shift towards the use of human tumor xenografts for the study of virtually all other types of anti-cancer drugs and treatment strategies.[1] Essentially every clinically approved anti-cancer drug was tested using these models, and showed positive anti-cancer effects before being evaluated in early, and then late phase clinical trials. Nevertheless, these successes have been overshadowed by highly visible failures in which a particular new anti-cancer drug, or treatment strategy, demonstrated remarkable

anti-tumor effects using a transplantable tumor model in mice, only to be followed by failure in the clinical trial setting[2] ("failure" in this case being defined here as having little or no survival benefit, regardless of whether it was found to be safe, or not, in humans).

Perhaps the most spectacular and recent example of this was the study by Boehm, O'Reilly et al.[3] who reported stunning effects of endostatin on three different transplantable tumors subcutaneously grown in syngeneic mice: the Lewis Lung carcinoma, the B16 melanoma and the T41 fibrosarcoma.[3] Cycles of daily endostatin treatment, an antiangiogenic protein drug, caused repeated and total regressions of established tumors. There was no evidence of relapse involving emergence of drug resistant variant/mutant subpopulations. Leaving aside the question of whether this result is reproducible (most other published studies of successful endostatin therapy show much more modest growth delays, but not overt tumor regressions), this result sparked enormous interest in both the scientific literature[4] and lay press.[5] It fueled unprecedented rapid initiation of phase I clinical trials in the United States, the results of which were recently reported.[6,7] The results of these trials showed the drug to be safe (which is the primary purpose of phase I trials) but there was certainly no evidence of the type of spectacular preclinical responses that had been observed in any of the treated patients.[6,7] The inevitable result has been the disappointment expressed not only about the drug itself, but about antiangiogenic therapy in general. In fairness, the results of other clinical trials involving antiangiogenic therapy such as the humanized monoclonal antibody to vascular endothelial cell growth factor (VEGF) known as bevacizumab (tradename: Avastin), which was tested in a randomized phase III trial as a third line therapy combined with Xeloda in advanced metastatic breast cancer, have also contributed significantly to this sense of current disappointment. But even in this case the disappointment stems, in part, from the many impressive results of prior preclinical studies utilizing a variety VEGF targeting of antiangiogenic drugs and approaches in a variety of mouse tumor models.

In 1999, Dr. Judah Folkman was quoted in a Newsweek magazine article as saying that a mouse study does not belong on the front page of the New York Times.[8] This makes considerable sense, and was a logical follow up to a quote he made in the May 3, 1998 Sunday New York Times article: "if you are a mouse and have cancer, we can take good care of you".[5] This statement would also seem to be logical, but as explained in this review, it is not necessarily so, and can be seriously challenged. Simply put, if you are a mouse with advanced, high-volume metastatic disease we probably cannot take good care of you.

The apparent lack of predictability of results often obtained using transplantable mouse or human tumors in normal or immune deficient mice has convinced many investigators to move away from such models and instead use spontaneously arising tumors, in particular genetically manipulated transgenic/knockout mice where the tumors which arise have mutations thought to be causative of the respective human cancers.[9,10] Alternatively, other investigators have suggested that transplantable tumor models can be made much more predictive by orthotopic transplantation which frequently facilitates metastatic spread—especially of human tumor xenografts[11,12]—and thus testing the effects of a given therapy on either (or both) the primary tumor growing in a physiologically relevant site (as opposed to an ectopic site) and distant metastatic disease.

In this commentary, two major points are made:

1. growth and testing of human tumors in subcutaneous tissue sites that are ectopic for a given type of cancer have provided relevant and predictive information to the clinic, provided that clinically relevant, pharmacokinetic parameters (especially dosing) are employed; and,
2. orthotopic transplants are nevertheless potentially valuable when used to generate metastases—but that therapy should be initiated at a point when the metastases are well established and macroscopic in nature (i.e., high volume metastatic disease)—and not just on low-volume (occult) minimum residual disease, which is what almost all previous studies have utilized when testing therapies on metastatic disease.

Also highlighted is the need for continuous vigilance with respect to the nature and origin of the cell lines used for transplantable tumor studies.

RETROSPECTIVE STUDIES OF CHEMOTHERAPEUTIC DRUGS USING SUBCUTANEOUS/ECTOPIC HUMAN TUMOR XENOGRAFTS SHOWING A HIGH DEGREE OF CLINICAL RELEVANCE

Nomura, Inaba and colleagues of the Cancer Chemotherapy Centre, Japanese Foundation for Cancer Research, Tokyo, have published a series of important and insightful studies which show clearly the remarkable potential of ectopic human tumor xenografts for predicting the pattern of activity of conventional cytotoxic chemotherapeutic drugs in humans.[13-17] Prior to undertaking their studies many other published reports showed that the majority of chemotherapeutic drugs have significant anti-tumor effects on a particular type of human cancer, even though most of the drugs tested were known not to have such activity on the respective tumor type in the clinical situation. In other words, the results of preclinical xenograft models were not retrospectively predictive of clinical activity. However, Nomura, Inaba and colleagues reasoned this could be due to inappropriate drug dosing. It turns out that the maximum tolerated dose (MTD) of most chemotherapeutic drugs that be given to mice is higher (4–5 times) than in humans. In some cases, the MTD is lower in mice than in humans, and in some cases (e.g., adriamycin) it is the same. Thus, in many cases, if one uses the MTD of a given chemotherapeutic drug for mice, the blood levels of drug will be significantly higher than can be attained in humans, leading to false positive tumor responses in mice.

To study this hypothesis, Nomura, Inaba and colleagues tested a large number of independent cell lines (e.g., generally eight to twelve) for each type of cancer tested. They reasoned this was similar in nature to the number of patients in a typical phase I clinical trial, and as such, would minimize the risks associated with obtaining a false positive or false negative response when testing just a single or few cell lines. In other words, one looks for an overall pattern of response in mice to different drugs that may be similar to what is seen a population of cancer patients. Each tumor cell line was grown as subcutaneous xenograft in a number of athymic nude mice. These mice were subsequently treated with at least 5 or 6 different chemotherapeutic drugs, tested as monotherapies, where some of the drugs were known to be clinically active on the particular type of human cancer being tested, and some not. The drugs were administered to some groups of tumor-bearing mice using the MTD of the drug for mice, whereas in another group the pharmacokinetically clinically equivalent dose (CED) or "rational dose" for humans was used.

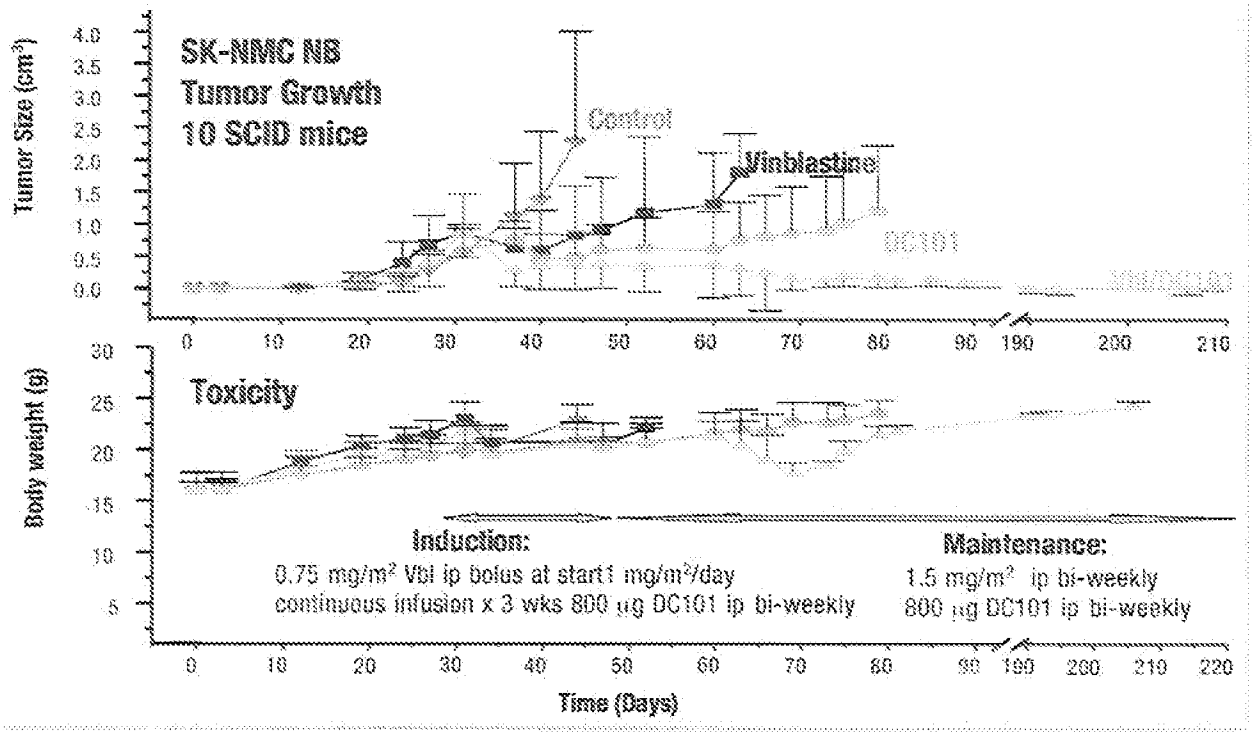

Figure 1. Results of an experiment in which large, established (0.75 cm³) human neuroblastomas (NB) were treated with a metronomic low-dose vinblastine schedule, or DC101 (an anti-VEGFR-2 monoclonal antibody) or a combination of the two drugs. The dosing of the drugs is indicated in the lower figure. Note that the metronomic/ maintenance regimen was preceded by an induction regimen of the same drug to try and rapidly debulk the tumor mass before initiating the metronomic low-dose chemotherapy schedule. Progression of disease was seen in the single treatment groups, whereas slow but eventually complete tumor regression was noted in the combination group in which the therapy was continued for 7 months, which was possible by the lack of toxicity of this regimen. Taken from Klement, G. et al. "Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity" *J Clin Invest* 2000; 105:R15-R24.

Analysis of the data for a large number of tumor types including lung, glioma, breast and gastric cancers showed that the pattern of response obtained when the mouse MTD was used was not associated with clinical pattern of responsiveness—most or all drugs showed activity. In other words, there was a high rate of false positives. In striking contrast, when the clinically equivalent or rational dose was used, the pattern of response in mice was similar to the activity of the respective drugs in the respective human cancer.[15-18]

These results were obtained using over 60 different established human cancer cell lines, all of which were injected subcutaneously. In no case was orthotopic injection of a cell line undertaken.

PROSPECTIVE STUDIES USING SUBCUTANEOUS HUMAN CHILDHOOD TUMOR XENOGRAFTS

Houghton and colleagues at St. Judes Children's Hospital in Memphis have also undertaken an exhaustive series of pharmacokinetic investigations in which a variety of pediatric malignancies were tested as subcutaneous xenografts in nude mice with respect to response to a variety of chemotherapeutic drugs. In particular, the relationship between systemic exposure and tumor response was evaluated, with emphasis on topoisomerase inhibitors such as irinotecan or topotecan.[19-26] These studies showed that a panel of neuroblastoma xenografts was highly sensitive to irinotecan, especially when administered using protracted schedules with lower doses of drug. For example, irinotecan was administered intravenously (i.v.) daily 5 days per week for 2 consecutive weeks (defined as one cycle) and compared to more protracted low-dose schedules where cycles were repeated every 21 days for a total of three courses. In the latter the total amount of drug was 5–10 mg/kg and was given using a daily schedule for 5 days, which was repeated 2 out of every 3 weeks for 9 weeks. Complete responses were observed in most of four of five xenografts using the intensive one cycle 40 mg/kg MTD schedule but the tumors tended to regrow. In contrast, with one exception, all neuroblastomas tested showed complete responses (CRs) which did not regrow during therapy when the protracted low-dose schedules were used involving a total dose of 10 mg/kg or 5 mg/kg.[23] Estimation of the lowest effective dose using the protracted i.v. schedule indicated that neuroblastomas respond to daily doses as low as 1.25 mg/kg.[23] It is interesting to consider these results in the light of those obtained by other investigators using a variety of similar protracted low-dose "metronomic" chemotherapy regimens as a putative antiangiogenic therapy, where increased efficacy and reduced toxicity have been frequently noted using such schedules, compared to the MTD of the same drug.[28-31]

The preclinical studies of Houghton and colleagues were directly translated to the clinic where the same protracted schedule was used and found to be well tolerated in children with refractory solid tumors; in addition encouraging, if not remarkable, rates of clinical responses were observed as well—16 of 33 patients experienced

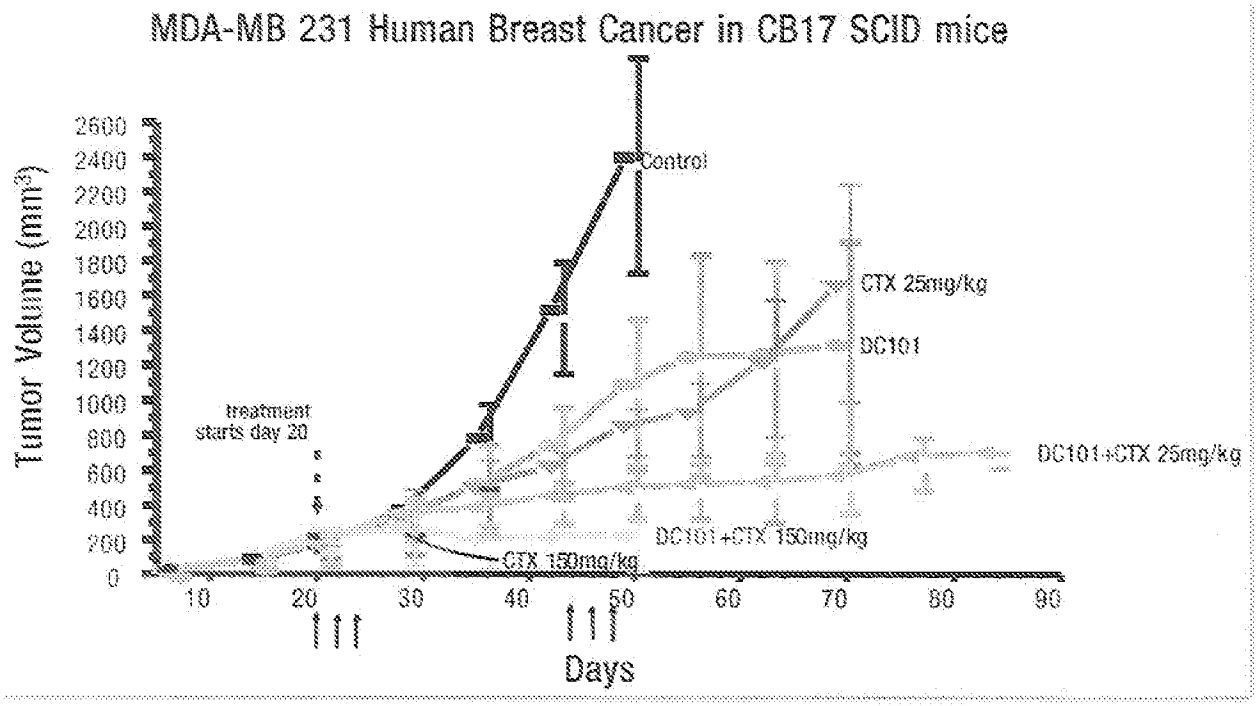

Figure 2. Results of an experiment published recently (Man et al. "Anti-tumor and anti-angiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water." Cancer Res., 62: 2731-2735, 2002) in which a human breast cancer cell line was injected "orthotopically" into the mammary fat pads of severe combined immunodeficient (SCID) mice, which allowed the tumor to metastasize to the lungs, liver and lymph nodes of the mice. Therapy was initiated when the "primary" intramammary fat pad tumor attained a size of 200 mm^3 and the disease had metastasized in a microscopic fashion only. Mice were then administered cyclophosphamide through their drinking water on a continuous non-stop basis at an estimated dose of 25 mg/kg per day, or treated with the DC101 anti-VEGFR-2 monoclonal antibody. In addition, another group of mice were given cyclophosphamide in the MTD fashion, i.e., at 150 mg/kg once every two days over a 6-day period (indicated by the vertical arrows). This MTD regimen was highly toxic to the SCID mice and resulted in death within one to two weeks. In contrast, mice given the same drug metronomically showed no signs of toxicity despite receiving up to 3 times the cumulative maximum tolerated dose given acutely.

stable disease and 5 showed partial responses.[24] These results show that preclinical xenograft models, even those involving ectopic/subcutaneous transplants, can provide useful predictors of the activity and responses of some pediatric cancers to topoisomerase I inhibitors such as irinotecan. A more detailed overview and discussion of the testing of new agents in childhood cancer models, both xenografts and transgenic oncomouse models was recently published by Houghton et al.[25]

IMPROVING HUMAN TUMOR XENOGRAFT MODELS FOR PREDICTING THE RELATIVE BENEFIT OF ANTI-CANCER DRUGS IN HUMANS—THE IMPORTANCE OF TREATING (ADVANCED) METASTATIC DISEASE

While the results summarized above are encouraging, and clearly show the potentially predictive value of human tumor xenografts, there is an aspect of the results in many of the preclinical studies that is nevertheless troubling: the excellent, if not remarkable, nature of the tumor responses in mice, as such responses are infrequently observed in cancer patients even though the drug being tested may be active against its respective human counterpart. For example, as discussed above, Houghton et al. observed complete responses of established solid neuroblastoma xenografts in a high proportion of cases using various irinotecan dosing schedules, especially protracted low-dose protocols.[24] However, such dramatic responses were not observed in the respective clinical trial of 23 patients, which included five children with neuroblastoma.[24] It is this aspect of experimental therapy studies in mice that can be frustrating as it often attracts considerable attention (e.g., the endostatin studies of Boehm, O'Reilly et al. discussed above) and expectation. This disparity has caused considerable skepticism about what to expect in the clinic on the basis of prior preclinical therapy studies. However, this skepticism may not always be justified when one takes into account, in retrospect, a crucial and fundamental difference between virtually all published experimental mouse therapy studies and corresponding clinical trials, and it is this: in most phase I, II and III clinical trials the patients being treated have advanced, high-volume metastatic disease whereas most mouse studies do not test the effects of therapy on advanced metastatic disease, but rather on a primary tumor transplant or spontaneously arising primary tumor, or microscopic, low-volume metastatic disease (Lee Ellis, personal communication). With respect to treatment of metastatic disease, typically, in such experiments, tumor cells are injected intravenously to generate lung or liver tumor colonies ("artificial metastases"), and therapy is initiated within one or a few days after injection of the cells—if not before tumor cell injection! This constitutes a form of adjuvant (or prophylactic) therapy, on microscopic, low-volume metastatic disease. Alternatively, growing primary tumors may be surgically removed, and treatment then initiated within a few days when the spontaneous metastases that have formed are microscopic in size. Thus, there is a much less demanding therapeutic situation for mice than for humans, when it

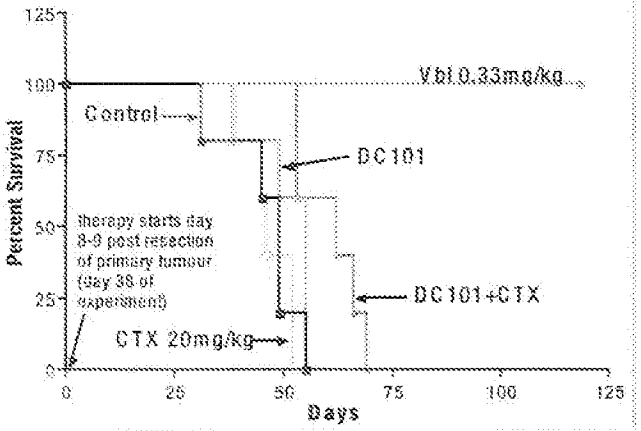

Figure 3. Effect of various therapy regimens on survival of SCID mice with advanced, metastatic cancer at the time of initiation of therapy. SCID mice were inoculated with MDA-MB-435 human tumor cells. The inoculation was into the mammary fat pads which facilitated distant metastatic spread, provided the primary tumors are surgically excised. This was done approximately 4 weeks after tumor cell inoculation, after which therapy was initiated approximately 8-9 days later. The cyclophosphamide was given continuously through the drinking water at an estimated dose of 20 mg/kg per day, whereas vinblastine or taxol at the indicated dose were injected at low-dose twice a week.

comes to comparing most preclinical trials to the clinical trial counterparts. Perhaps much of the disparity in results between the two is related to this variable since it is well known that high-volume advanced metastatic disease is generally much more difficult to treat than low-volume adjuvant disease. Add to this the fact that many patients entered into clinical trials had been treated previously with other therapies and have relapsed with refractory disease. Heavily pretreated and resistant patients are often less responsive to a new therapy, and usually have advanced metastatic disease at the time of entry into a clinical trial.[35] How often have investigators in the past tested a new drug or therapy in mice where this dire clinical situation is recapitulated? The answer is rarely—if ever.

To illustrate the point about treating (advanced) metastatic disease, some recent results obtained in this laboratory are shown. Figure 1 shows the results of an experiment in which a metronomic low-dose vinblastine protocol, in combination with an antiangiogenic drug, called DC101 (an anti-VEGF receptor-2 blocking antibody) was used to treat large, established human neuroblastoma xenografts obtained after subcutaneous injection of SK-NM-C cells.[27] The results showed a remarkable anti-tumor effect could be obtained with the combination—sustained and complete tumor regressions. In effect, the mice were cured since the therapy was continuously maintained for 7 months,[27] and surprisingly, tumors did not resume growth when the treatment was finally terminated (unpublished observations). However, because the tumors were injected subcutaneously (i.e., ectopically) they did not metastasize, and therefore the much more demanding clinical situation of treating advanced metastatic neuroblastoma metastases was not duplicated in the mouse studies. The preclinical study was not intended to predict clinical activity—as implied by a headline proclaimed on the front page of a prominent national Canadian newspaper,[36] but to illustrate the principle of metronomic low-dose chemotherapy as a relatively non-toxic and effective way of giving chemotherapy, and combining it with a targeted antiangiogenic drug.[27,35-38]

Figure 2 shows the results of a similar experiment in which a human breast cancer (MDA-MB-231) was injected orthotopically in the mammary fat pads of female SCID mice, and then treated continuously with an oral low-dose regimen of cyclophosphamide administered continuously through the drinking water, combined with the same antiangiogenic drug, DC101.[29] A control using an MTD regimen of cyclophosphamide was also used. In terms of survival, the best treatment regimen was the combination of the metronomic oral low-dose cyclophosphamide/ DC101, and the survival benefit was obvious. However, in this model, while the orthotopic breast cancer can metastasize, the metastases remain largely microscopic because of the retention of the primary tumor and the timing of the initiation of treatment. Thus, treatment of low-volume, metastatic disease was undertaken.

More recent experiments have involved 'raising the therapeutic bar', so to speak. In Figure 3 a tumor cell line, called MDA-MB-435, supposedly a well known breast cancer cell line used extensively in breast cancer research, was injected into the mammary fat pads of SCID mice and allowed to grow for about one month. The resultant primary tumors were then surgically removed and initiation of treatment with oral low-dose cyclophosphamide and/or DC101 was delayed for about 10 days to allow establishment of extensive macroscopic metastases in the lungs and draining lymph nodes of the SCID mice, as well as diffuse metastatic spread in the liver (data not shown). Using survival as an endpoint, neither DC101 alone or oral low-dose cyclophosphamide alone had had impact on survival; the combination did have an effect, but the magnitude of the benefit was rather modest in comparison to the sort of results shown in Figures 1 and 2. Of considerable interest, however, was the finding that a metronomic low-dose vinblastine protocol—0.33 mg/kg given intraperitoneally three times a week—alone caused complete resolution of advanced metastatic disease and greatly prolonged survival of the mice. Eventually, the mice had to be sacrificed because tumors recurred at the site of surgical removal and grew progressively in spite of the success of the therapy on distant metastatic disease (unpublished observations).

It is of course difficult to compare the results of each experiment since different tumor cell lines and different treatment regimens were used. Indeed, the MDA-MB-435 'breast' tumor cell line has recently been implicated to be a melanoma, based on gene and protein expression profiling,[39,40] results which we have confirmed using the MDA-MB-435 line discussed in Figure 3. Nevertheless, the results of Figure 3 do suggest that treatment of advanced metastatic disease in mice will give results that may turn out to be much more reflective, i.e., predictive, of the clinical situation typically encountered when testing new drugs in phase I, II or III clinical trials. The vinblastine therapy results also point to the possibility that we cannot always assume that the response of a primary tumor will mirror the effects of the same therapy on distant metastases—this is obvious. What is not so obvious, and surprising, is that the response of metastases may be significantly better than the primary tumor in some cases. We would anticipate that this would be the exception rather than the rule; nevertheless this has ramifications for anti-cancer screening and drug testing, if correct.

CONCLUSIONS

In light of these results one might want to rethink Dr. Folkman's quote "if you are a mouse and have cancer, we can take good care of you".[5] One may argue this applies to mice with rapidly growing, transplanted, subcutaneous, encapsulated/non-metastatic tumors. In contrast, mice with high-volume, advanced, metastatic disease in sites such as the lungs, liver and brain may not be so easy to take care of, similar to their human counterparts. The vinblastine results do however provide some basis for optimism, and emphasize the need to begin testing models which involve advanced metastatic disease. This, incidentally, is one of the limitations of many of the current transgenic oncomouse models, as they usually do not spontaneously metastasize.[41,42] Moreover, monitoring the effects of therapies on metastatic disease in mice is becoming easier and less subjective with the growing use of small animal non-invasive micro-imaging research tools[43] and non-invasive biochemical techniques, e.g., measuring secreted tumor-specific protein markers that can be introduced into tumor cell lines.[29,44] It is also time to reexamine some of the current dogmas regarding mouse models of cancer. First, human tumor xenografts can be surprisingly predictive of clinical activity, and in some cases this includes subcutaneous/ectopic transplants. The wisdom of the rush towards exclusive use of much more expensive transgenic oncomouse models for drug therapy testing can be questioned, especially when such tumors fail to express the most critical element of malignant disease—ability to metastasize, and the fact that less expensive transplantable tumor models are available which work—if used appropriately.

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References

1. Shimosato Y, Kameya T, Nagai K, Hirohashi S, Koide T, Hayashi H, Nomura T. Transplantation of human tumors in nude mice. J Natl Cancer Inst 1976; 56:1251-60.
2. Takimoto CH. Why drugs fail: of mice and men revisited. Clin Cancer Res 2001; 7:229-30.
3. Boehm T, Folkman J, Browder T, and O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 1997; 390:404-7.
4. Kerbel RS. A cancer therapy resistant to resistance. Nature 1997; 390:335-6.
5. Kolata G. A cautious awe greets drugs that eradicate tumors in mice. The New York Times: May 3, 1998.
6. Twombly R. First clinical trials of endostatin yield lukewarm results. J Natl Cancer Inst 2002; 94:1520-1.
7. Eder JP Jr, Supko JG, Clark JW, Puchalski TA, Garcia-Carbonero R, et al. Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. J Clin Oncol 2002; 20:3772-84.
8. Kalb C. Hype, hope, cancer. Newsweek, 73, 12-28-1998.
9. Jackson-Grusby L. Modeling cancer in mice. Oncogene 2002; 21:5504-14.
10. Van Dyke T, Jacks T. Cancer modeling in the modern era: progress and challenges. Cell 2002; 108:135-44.
11. Fidler IJ. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. Cancer Metastasis Rev 1986; 5:29-49.
12. Hoffman RM. Orthotopic transplant mouse models with green fluorescent protein-expressing cancer cells to visualize metastasis and angiogenesis. Cancer Metastasis Rev 1998; 17:271-7.
13. Mattern K, Uesugi Y, Inaba M, Fiebig H, Ohnishi Y, Nakamura O, et al. Responsiveness of subcutaneous human glioma xenografts to various antitumor agents. Anticancer Res 1990; 10:309-13.
14. Inaba M, Tashiro T, Kobayashi T, Sakurai Y, Maruo K, Ohnishi Y, et al. Responsiveness of human gastric tumors implanted in nude mice to clinically equivalent doses of various antitumor agents. Jpn J Cancer Res 1988; 79:517-22.
15. Inaba M, Kobayashi T, Tashiro T, Sakurai Y, Maruo K, Ohnishi Y, et al. Evaluation of antitumor activity in a human breast tumor/nude mouse model with a special emphasis on treatment dose. Cancer 1989; 64:1577-82.
16. Tashiro T, Inaba M, Kobayashi T, Sakurai Y, Maruo K, Ohnishi Y, et al. Responsiveness of human lung cancer/nude mouse to antitumor agents in a model using clinically equivalent doses. Cancer Chemother Pharmacol 1989; 24:187-92.
17. Inaba M, Kobayashi T, Tashiro T, Sakurai Y. Pharmacokinetic approach to rational therapeutic doses for human tumor-bearing nude mice. Jpn J Cancer Res 1988; 79:509-16.
18. Thompson J, Zamboni WC, Cheshire PJ, Richmond L, Luo X, Houghton JA, et al. Efficacy of oral irinotecan against neuroblastoma xenografts. Anticancer Drugs 1997; 8:313-22.
19. Vassal G, Boland I, Santos A, Bissery MC, Terrier-Lacombe MJ, Morizet J, et al. Potent therapeutic activity of irinotecan (CPT-11) and its schedule dependency in medulloblastoma xenografts in nude mice. Int J Cancer 1997; 73:156-63.
20. Kirstein MN, Houghton PJ, Cheshire PJ, Richmond LB, Smith AK, Hanna SK, Stewart CF. Relation between 9-aminocamptothecin systemic exposure and tumor response in human solid tumor xenografts. Clin Cancer Res 2001; 7:358-66.
21. Zamboni WC, Stewart CF, Thompson J, Santana VM, Cheshire PJ, Richmond LB, et al. Relationship between topotecan systemic exposure and tumor response in human neuroblastoma xenografts. J Natl Cancer Inst 1998; 90:505-11.
22. Houghton PJ, Cheshire PJ, Hallman JD, Lutz L, Friedman HS, Danks MK, Houghton JA. Efficacy of topoisomerase I inhibitors, topotecan and irinotecan, administered at low dose levels in protracted schedules to mice bearing xenografts of human tumors. Cancer Chemother Pharmacol 1995; 36:393-403.
23. Thompson J, Zamboni WC, Cheshire PJ, Lutz L, Luo X, Li Y, et al. Efficacy of systemic administration of irinotecan against neuroblastoma xenografts. Clin Cancer Res 1997; 3:423-31.
24. Furman WL, Stewart CF, Poquette CA, Pratt CB, Santana VM, Zamboni WC, et al. Direct translation of a protracted irinotecan schedule from a xenograft model to a phase I trial in children. J Clin Oncol 1999; 17:1815-24.
25. Browder T, Butterfield CE, Kraling BM, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 2000; 60:1878-86.
26. Bello L, Carrabba G, Giussani C, Lucini V, Cerutti F, Scaglione F, et al. Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo. Cancer Res 2001; 61:7501-6.
27. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin D, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 2000; 105:R15-24.
28. Klement G, Mayer B, Huang P, Man S, Bohlen P, Hicklin D, et al. Differences in therapeutic indexes of combination metronomic chemotherapy and an anti-VEGFR-2 antibody in multidrug resistant human breast cancer xenografts. Clin Cancer Res 2002; 8:221-32.
29. Man S, Bocci G, Francia G, Green S, Jothy S, Bergers G, et al. Antitumor and anti-angiogenic effects in mice of low-dose (metronomic) cyclophosphamide administered continuously through the drinking water. Cancer Res 2002; 62:2731-5.
30. Gately S, Kerbel R. Antiangiogenic scheduling of lower dose cancer chemotherapy. Cancer J 2001; 7:427-36.
31. Gasparini G. Metronomic scheduling: the future of chemotherapy? Lancet Oncology 2001; 2:733-740.
32. Houghton PJ, Adamson PC, Blaney S, Fine HA, Gorlick R, Haber M, et al. Testing of new agents in childhood cancer preclinical models: meeting summary. Clin Cancer Res 2002; 8:3646-57.
33. Horowitz ME, Etcubanas E, Christensen ML, Houghton JA, George SL, Green AA, Houghton PJ. Phase II testing of melphalan in children with newly diagnosed rhabdomyosarcoma: a model for anticancer drug development. J Clin Oncol 1988; 6:308-14.
34. Bergsma B. In mice and men, less can be more. National Post, April 1, 2000.
35. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 2000; 105:1045-7.
36. Barinaga M. Angiogenesis research: cancer drugs found to work in new way. Science 2000; 288:245.
37. Fidler IJ, Ellis LM. Chemotherapeutic drugs—more really is not better. Nature Medicine 2000; 6:500-2.
38. Nathan SA, Rubin E, Aisner J, Clarusso E. High-dose chemotherapy or high dose for low dose. J Clin Oncol 2000; 18:2945-7.
39. Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, et al. Systematic variation in gene expression patterns in human cancer cell lines. Nat Genet 2000; 24:227-35.
40. Ellison G, Klinowska T, Westwood RF, Docter E, French T, Fox JC. Further evidence to support the melanocytic origin of MDA-MB-435. Mol Pathol 2002; 55:294-9.
41. Kerbel RS. What is the optimal rodent model for anti-tumor drug testing? Cancer Metastasis Rev 1998; 17:301-4.
42. Rosenberg MP, Bortner D. Why transgenic and knockout animal models should be used (for drug efficacy studies in cancer). Cancer Metastasis Rev 1998; 17:295-9.
43. Hoffman R. Green fluorescent protein imaging of tumour growth, metastasis, and angiogenesis in mouse models. Lancet Oncol 2002; 3:546-56.
44. Shih IM, Torrance C, Sokoll LJ, Chan DW, Kinzler KW, Vogelstein B. Assessing tumors in living animals through measurement of urinary beta-human chorionic gonadotropin. Nature Medicine 2000; 6:711-4.